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14. ABSTRACT
This project aimed at characterization of NF1 protein ubiquitination system by identifying ubiquitination sites and ligases for neurofibromin. We established the method for obtaining ubiquitinated neurofibromin for mass-spectrometric sequencing and identification of ubiquitination sites of neurofibromin. We confirmed neurofibromin ubiquitination and degradation after mitogenic stimulation by serum and LPA. Cbl-Ring finger ubiquitin ligase mediates ubiquitination and degradation of a variety of signaling proteins in the tyrosine kingase receptor signaling pathway. As one of efforts for identifying neurofibromin ubiquitin ligases, we discovered that ubiquitination and degradation of neurofibromin was induced by Cbl which may be responsible for neurofibromin ubiquitination upon activation of tyrosine-kinase receptors.

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Neurofibromin ubiquitination, ubiquitination sites, mass-spectrometric sequencing, ubiquitin ligases, Cbl Ring finger ubiquitin ligase

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INTRODUCTION

Loss of NF1 tumor suppressor activity is induced by instability of NF1 protein that is mediated by ubiquitination-dependent degradation [1]. In this system, ubiquitin-conjugation to particular lysine residues of protein triggers its degradation. The specificity of ubiquitination is determined by ubiquitin protein ligases. Therefore, this project was designed to identify ubiquitination sites and ligases for neurofibromin.

BODY

Task 1: To identify ubiquitination site(s) of neurofibromatosis type I (NF1) protein

1a. Preparation of ubiquitinated neurofibromin for mass-spectrometric sequence analysis of ubiquitination sites and types

Ubiquitination of NF1 protein was induced by re-feeding after starving 293 cells. To obtain ubiquitinated-NF1 protein enough for mass-spectrometric sequencing analysis, 293 cells were pretreated with proteasome inhibitor, MG132, before re-feeding.

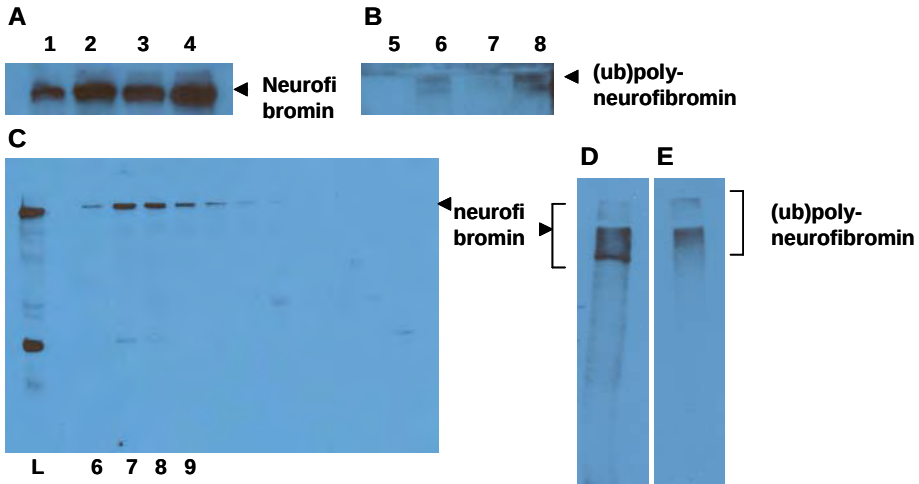

Fig.1 Preparation of ubiquitinated neurofibromin

A Nuerofibromin protein level in growing (1), 15 (2) or 5 (3) min-refed 293 cells after starvation (4). **B** Ubiquitinated neurofibromin identified by anti-ubiquitin antibody on anti-neurofibromin IP from growing (6), starved (7), or 15 min-refed 293 cells after starvation (8). (5) control IP with agarose beads. **C** Neurofibromin protein identified in FPLC-Superose 12 column fractions of 293 lysate refed for 15 min after starvation overnight. Lane L: lysate. Peak fractions (7-9) were combined and subjected to IP. WB of IP with anti-neurofibromin (D) and anti-ubiquitin (E).

The lysates were prepared from 293 cells in the growing phase, after starvation overnight, or refed with 10% serum for 5 or 15 min after starvation. Neurofibromin protein level in those lysates was analyzed by Western Blot using anti-neurofibromin antibody. As shown in Fig.1A, neurofibromin protein increased after starvation (Lane 4 vs 1) and decreased following serum addition (compare Lane 4 to 2 (5 min) and 3 (15 min), as reported previously [1]. Under similar condition, ubiquitinated-neurofibromin was analyzed by immunoprecipitation with anti-neurofibromin antibody (Santa Cruz, CA) and Western Blotting. Fig.1B shows that ubiquitinated neurofibromin was detected after re-

feeding of starved cells (Lane 8), whereas no ubiquitinated form was detected in starved, quiescent cells (Lane 7).

To maximize the recovery of ubiquitinated-neurofibromin, 293 cells from 10 plates were starved overnight and re-fed for 15 min after treatment with MG132 (proteasome inhibitor) which blocked degradation and increased ubiquitination. The lysate was applied on FPLC-Superose 12 gel filtration chromatography (Fig.1C). The peak neurofibromin fractions were pooled and subjected to immunoprecipitation using anti-neurofibromin antibody. The IP was analyzed by Western Blotting using anti-neurofibromin (Fig.1D) and anti-ubiquitin (Fig.1E), and showed unmodified and ubiquitinated neurofibromin in the near to the top and as a smear down from the top of the gel, respectively. By CBB protein staining of polyacrylamide gels which were run in parallel (data not shown), the protein band, corresponding to unmodified neurofibromin, was cut out as a control neurofibromin. Above this band, several protein bands were seen and combined as neurofibromin possibly containing several ubiquitin molecules. The band on the top of gel was separately cut out as poly-ubiquitinated neurofibromin. These gel bands are processed for sequence analysis by mass-spectrometry. However, the quantity was not enough for identifying ubiquitination sites. Since the quality of the preparation was good enough for mass-spectrometric analysis, we are in process of scaling up for determination of ubiquitination sites.

Task 2: To identify ubiquitin ligase for NF1 protein

In our grant proposal, we planned two methods; **Method 1** to isolate NF1-ubiquitin ligase from GST-NF1 protein-interacting proteins and **Method 2** to isolate NF1-ubiquitin ligase by conventional column chromatography. Since we do not know ubiquitin ligase-binding domain in NF1, we realized it rather time-consuming to clone a construct expressing a whole length of the *NF1* gene (~300kbs).

We have set to identify and isolate NF1-ubiquitin ligase by conventional column chromatography. Since NF1-ubiquitination/degradation was shown to be induced by tyrosine kinase- and G-protein-coupled receptor pathways previously [1], we first confirmed the results. Since tyrosine-kinase receptor pathway utilizes Cbl ubiquitin ligase, we tested if Cbl is involved in NF1 ubiquitination and degradation.

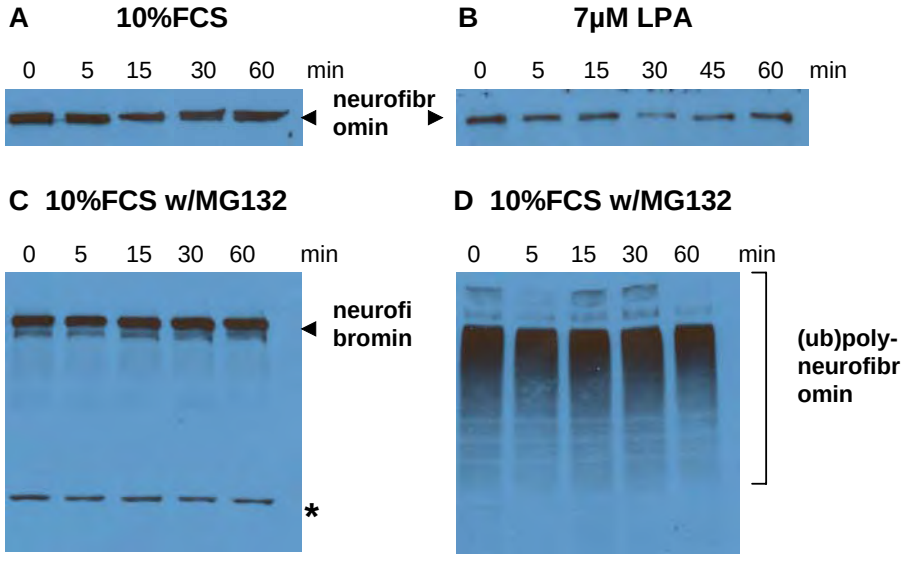

Fig.2 Degradation and ubiquitination of neurofibromin by serum and LPA

Neurofibromin is analyzed with time after addition of serum (**A**) or LPA (**B**) to starved 293 cells. **C** Similar analysis was performed with serum addition, but cells were pre-treated with 20μM MG132, proteasome inhibitor, for 30 min before serum addition. * appears to be degradation product of neurofibromin. **D** IP with anti-neurofibromin was prepared for each lysate for Panel C. IP's were analyzed by Western Blot using anti-ubiquitin antibody.

2a. Induction of proteasomal degradation of neurofibromin by serum and LPA

To confirm the ubiquitin/proteasome-mediated pathway for neurofibromin down-regulation, the kinetics of neurofibromin change were analyzed following mitogenic stimulation of quiescent 293 cells in which neurofibromin was elevated. Quiescent cells were obtained by

culturing in the serum-free medium overnight, as described. Following the addition of 10% FCS or 7μM LPA, cells were collected for the analysis of neurofibromin by Western blotting With 10% FCS, neurofibromin protein reached at the lowest in 15 min, although the reduction was moderate (Fig.2A). On the other hand, LPA induced a more significant, sustained decrease of neurofibromin protein, reaching the lowest level after 30 min (Fig.2B). These kinetics after mitogenic stimulation by serum and LPA are similar to those reported previously.

To confirm that the neurofibromin degradation is mediated by ubiquitin/proteasome pathway, neurofibromin level was similarly analyzed after the addition of 10% FCS to quiescent cells which had been pretreated for 30 min with MG132 (proteasome inhibitor) to block degradation. As shown in Fig.2C, neurofibromin remained at the same level for 1 hour after addition of 10% FCS. To examine ubiquitination, the extracts were also subjected to IP with anti-neurofibromin antibody, followed by Western blotting analysis using anti-ubiquitin antibody. Fig.2D shows that neurofibromin ubiquitination remains at a high level, with no change in ubiquitination for 1 hour.

Thus, these results appear to confirm that mitogen-induced degradation of neurofibromin is mediated by ubiquitin/proteasome pathway, as reported [1]. Serum induces a more rapid, moderate degradation, followed by rapid recovery, whereas LPA induces a slower response, but a more sustained, significant degradation. These different kinetics may indicate that distinct ubiquitin conjugation/deconjugation systems are involved in mediating different signaling pathway for each type of growth factors. Therefore, multiple types of ubiquitin ligase and/or deubiquitinating enzyme may mediate neurofibromin ubiquitination, depending on the signal transmitted.

2b. **Involvement of Cbl ubiquitin ligase in neurofibromin ubiquitnation and degradation**

Stimulation of tyrosin kinase receptors was shown to induce ubiquitin/protesome-mediated degradation of neurofibromin [1]. Upon ligand binding to receptor, EGF receptor and other signaling proteins are targeted by Cbl ubiquitin ligase. Therefore, we tested the effect of Cbl-overexpression on degradation and ubiquitination of neurofibromin.

The Cbl family proteins consists of the N-terminal tyrosine kinase binding domain, RING-finger ubiquitin ligase motif, proline-rich region, and C-terminal ubiquitin-associated (UBA) domain that overlaps with a leucine-zipper (LZ) motif. Three mammalian forms, c-Cbl, Cbl-b and Cbl-c, are different in the C-terminal length. Cbl-c, a shorter isoform, lacks the C-terminal UBA/LZ domain.

Neurofibromin protein level was examined in 293 cells over-expressing increasing concentrations of Cbl-c. The effect of Cbl-overexpression was examined in growing or quiescent cells. As shown in Fig.3A, Cbl-overexpression reduced neurofibromin in the growing cells, but did not in quiescent cells. The increased degradation was associated with a slight increase of ubiquitination, as shown by Western blotting analysis using anti-ubiquitin antibody on IP with anti-neurofibromin antibody (Fig.3B). These very low levels of ubiquitination detected is due to the general difficulty of detecting ubiquitination, without proteasome inhibitors, due to efficient deubiquitnating activities in the extract. These results show that Cbl-overexpression induces neurofibromin degradation only in the proliferating cells. They may therefore suggest that distinct regulatory mechanisms operate to sustain at a high level in the quiescent cells or to induce degradation in mitogenic cells, for example by induction of DUB in quiescent cells so that Cbl expression was counteracted by a high level of DUB.

The GRD-domain of human neurofibromin (1064-1761aas), tagged with (His)6, was constructed (His-GRD). Mutation (K1105R) was previously shown to reduce the degradation of neurofibromin. Using site-directed PCR-mediated mutagenesis, K1105R mutation was introduced to His-GRD. These wild type and mutant GRD's were expressed along with Cbl-b or c. The level of GRD's was analyzed by Western blotting. Fig.3C shows that the degradation of both wild type and mutant GRD's was similarly induced by Cbl. Ubiquitination of GRD, both wild type and K-R mutant, appears to be also elevated slightly. Therefore, these results suggest that neurofibromin is ubiquitinated at multiple sites,

which coordinately induce degradation and that the deletion of ubiquitination at a single site shows a minimal effect. This type and function of ubiquitination are generally seen with a variety of proteins. In addition, the degradation of GRD with the same mutation was partially inhibited in the study reported previously.

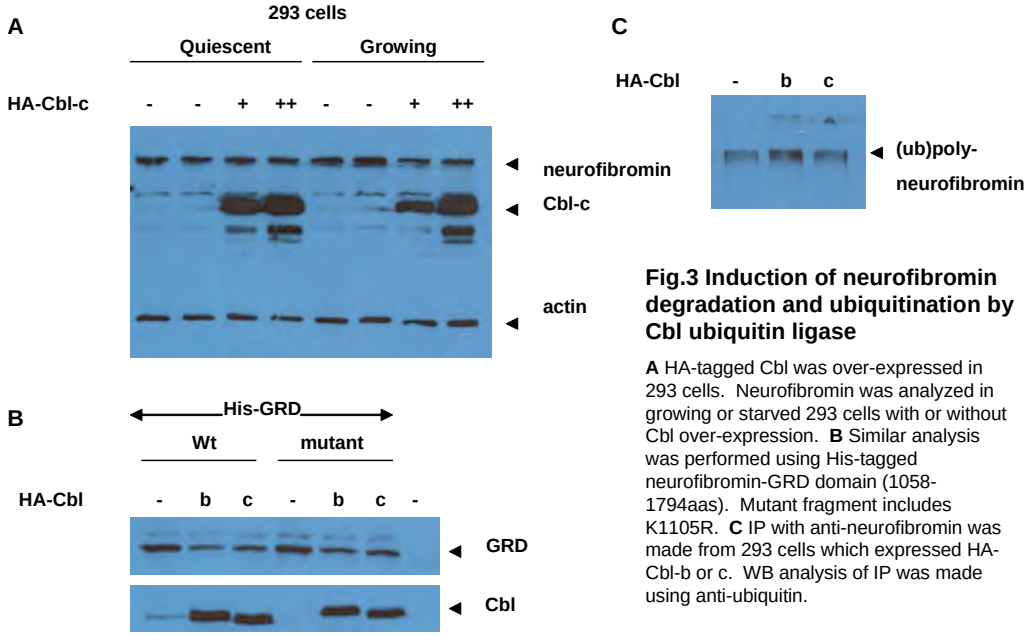

Fig.3 Induction of neurofibromin degradation and ubiquitination by Cbl ubiquitin ligase

A HA-tagged Cbl was over-expressed in 293 cells. Neurofibromin was analyzed in growing or starved 293 cells with or without Cbl over-expression. **B** Similar analysis was performed using His-tagged neurofibromin-GRD domain (1058-1794aas). Mutant fragment includes K1105R. **C** IP with anti-neurofibromin was made from 293 cells which expressed HA-Cbl-b or c. WB analysis of IP was made using anti-ubiquitin.

Thus, Cbl ubiquitin ligase may be responsible for neurofibromin ubiquitination and degradation in the tyrosine-kinase receptor pathway. The GRD domain appears to contain the functional domains for Cbl ligase activity. We plan to further investigate the function of Cbl ligase for neurofibromin protein regulation in the tyrosin kinase-receptor signaling.

Ubiquitin-affinity chromatography has been effectively utilized for isolation of ubiquitnation systems. We are in process of applying this system for identification and isolation of NF1-ubiquitination systems which are responsible for tyrosine-kinase and G-protein coupled receptor pathways.

Key Research Accomplishment

Task 1

a. We established the method for isolation of ubiquitinated neurofibromin enough for identification of ubiquitination sites by mass-spectrometry, including induction of neurofibromin ubiquitination, inhibition of de-ubiquitinating enzymes, and FPLC-column chromatography
b. We established the method for mass-spectrometric determination of neurofibromin ubiquitination sites.

Task 2

a. We identified the kinetics of neurofibromin changes after different mitogenic stimulations and suggested an involvement of different ubiquitin ligases for different mitogens.
b. We identified that Cbl-RING domain ligase, that mediated ubiquitination and degradation of proteins down-stream of tyrosine kinase receptors, induced degradation and ubiquitination of neurofibromin.

c. We identified that Cbl catalyzes ubiquitination of the GRD region of neurofibromin, suggesting that this region may have an interacting domain with Cbl.

Reportable outcomes

Task 1

We are processing a large volume of 203 cells for determination of neurofibromin ubiquitination sites. The results will be submitted as soon as sites will be determined.

Task 2

Before publication on the function of Cbl ligase on neurofibromin ubiquitination, we will determine the function of Cbl ubiquitin ligase for neurofibromin degradation after EGF stimulation by use of siRNA specific to Cbl. We will further characterize a binding domain with Cbl by construction for the expression of neurofibromin domains, particularly the GRD domain.

Conclusions

We achieved both tasks 1 and 2, although time was limited to make publishable data. We will continue working on both tasks with the goal of developing therapeutics using the specificities of ubiquitination sites and neurofibromin ubiquitin ligases.

References

1. Ciechowski K, Santiago S, Jardim M, Johnson BW, Jacks T: Dymanic regulation of the Ras pathway via proteolysis of the NF1 tumor suppressor. Genes & Dev 17:449-454, 2003

Appendix

None